

TRANSMITTAL LETTER TO THE UNITED STATES
DESIGNATED/ELECTED OFFICE (DO/EO/US)
CONCERNING A FILING UNDER 35 U.S.C. 371

ATTORNEY'S DOCKET NUMBER

1038-1130 MIS:jb

U.S. APPLICATION NO. (IF KNOWN, SEE 37 CFR

09/786751

INTERNATIONAL APPLICATION NO.
PCT/CA99/00917INTERNATIONAL FILING DATE
October 4, 1999PRIORITY DATE CLAIMED
October 5, 1998TITLE OF INVENTION
QUANTIFICATION OF RNAAPPLICANT(S) FOR DO/EO/US
Mark Parrington; et al.

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. ☐ This is an express request to begin national examination procedures (35 U.S.C. 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39(1).
4. ☐ A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.
5. ☒ A copy of the International Application as filed (35 U.S.C. 371 (c) (2))
 - a. ☐ is transmitted herewith (required only if not transmitted by the International Bureau).
 - b. ☒ has been transmitted by the International Bureau.
 - c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US).
6. ☐ A translation of the International Application into English (35 U.S.C. 371(c)(2)).
7. ☐ A copy of the International Search Report (PCT/ISA/210).
8. ☐ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371 (c)(3))
 - a. ☐ are transmitted herewith (required only if not transmitted by the International Bureau).
 - b. ☐ have been transmitted by the International Bureau.
 - c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
 - d. ☐ have not been made and will not be made.
9. ☐ A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
10. ☒ An oath or declaration of the inventor(s) (35 U.S.C. 371 (c)(4)). - unsigned copy
11. ☒ A copy of the International Preliminary Examination Report (PCT/IPEA/409).
12. ☐ A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371 (c)(5)).

Items 13 to 20 below concern document(s) or information included:

13. ☐ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
14. ☐ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
15. ☒ A **FIRST** preliminary amendment.
16. ☐ A **SECOND** or **SUBSEQUENT** preliminary amendment.
17. ☐ A substitute specification.
18. ☐ A change of power of attorney and/or address letter.
19. ☐ Certificate of Mailing by Express Mail
20. ☐ Other items or information:

09/7786731

PCT/CA99/00917

1038-1130 MIS:jb

21. The following fees are submitted:

BASIC NATIONAL FEE (37 CFR 1.492 (a) (1) - (5)) :

- ☒ Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO **\$970.00**
- ☐ International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO **\$840.00**
- ☐ International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2)) paid to USPTO **\$690.00**
- ☐ International preliminary examination fee paid to USPTO (37 CFR 1.482) but all claims did not satisfy provisions of PCT Article 33(1)-(4) **\$670.00**
- ☐ International preliminary examination fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Article 33(1)-(4) **\$96.00**

ENTER APPROPRIATE BASIC FEE AMOUNT =**\$970.00**

Surcharge of **\$130.00** for furnishing the oath or declaration later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492 (e)).

\$0.00

CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE	
Total claims	14 - 20 =	0	x \$18.00	\$0.00
Independent claims	3 - 3 =	0	x \$78.00	\$0.00
Multiple Dependent Claims (check if applicable).				<input type="checkbox"/> \$0.00

TOTAL OF ABOVE CALCULATIONS =**\$970.00**

Reduction of 1/2 for filing by small entity, if applicable. Verified Small Entity Statement must also be filed (Note 37 CFR 1.9, 1.27, 1.28) (check if applicable).

☐**\$0.00****SUBTOTAL =****\$970.00**

Processing fee of **\$130.00** for furnishing the English translation later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492 (f)).

+

\$0.00**TOTAL NATIONAL FEE =****\$970.00**

Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31) (check if applicable).

☐**\$0.00****TOTAL FEES ENCLOSED =****\$970.00**

Amount to be:	\$
refunded	
charged	\$

☒ A check in the amount of **\$970.00** to cover the above fees is enclosed.

☐ Please charge my Deposit Account No. _____ in the amount of _____ to cover the above fees.
A duplicate copy of this sheet is enclosed.

☒ The Commissioner is hereby authorized to charge any fees which may be required, or credit any overpayment to Deposit Account No. **19-2253** A duplicate copy of this sheet is enclosed.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO:

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24223

PATENT, TRADEMARK OFFICE

SIGNATURE

Michael I. Stewart

NAME

24,973

REGISTRATION NUMBER

March 7, 2001

DATE

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re National Phase of International Application:

Appl'n. No. : PCT/CA99/00917
Filed : October 4, 1999
Applicant : Mark Parrington, et al.
Title : QUANTITATION OF RNA
Docket No. : 1038-1130 MIS:jb

March 7, 2001

BY COURIER

The Commissioner of Patents
and Trademarks,
Washington, D.C. 20231,
U.S.A.

PRELIMINARY MENDMENT

Sir:

Please amend the above-identified application as follows:

In the Specification:

Before the first line of the specification, add the following:

" **REFERENCE TO RELATED APPLICATIONS**

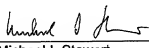
This application is a national phase application under 35 U.S.C. 371 of PCT/CA99/00917."

REMARKS/ARGUMENTS

The specification has been amended on page 1 to reflect that this application is a U.S. National Phase filing under 35 U.S.C. 371 of PCT/CA99/00917.

Attached hereto is a marked-up version of the changes made to the specification and claims by the current amendment. The attached page is captioned "**Version with markings to show changes made.**"

Respectfully submitted,


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Appl. No. National Phase of PCT/CA99/00917

VERSION WITH MARKINGS TO SHOW CHANGES MADE

In the Specification:

Paragraph beginning at line 3 of page 1 has been amended as follows:

REFERENCE TO RELATED APPLICATIONS

_____ This application is a national phase application under 35 U.S.C. 371 of
PCT/CA99/00917.

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TITLE OF THE INVENTIONQUANTITATION OF RNAFIELD OF INVENTION

The present invention relates to the quantitation
5 of RNA present in tissue, thereby permitting analysis,
for example, of rare transcript expression in cells.

BACKGROUND OF THE INVENTION

It is known in the art how to amplify DNA by
polymerase chain reaction (PCR). It has also been
10 shown that mRNA can be transcribed into DNA templates
and then amplified by PCR in a method known as RT-PCR.
The limitation of existing RT-PCR methods is that some
very rare transcripts (mRNAs) are unable to be
amplified in the RT and subsequent PCR portion of the
15 existing methods. It would be a great benefit in the
analysis of subtle changes in the expression levels of
certain genes, for example, cytokine genes in non-
lymphode tissue, to be able to not only to detect but
quantify the levels of these transcripts after
20 treatment of the host.

Previously RT-PCR was performed in two distinct
steps. This involved the reverse transcriptase step in
which mRNA was transcribed into DNA mediated by the
enzyme reverse transcriptase. This enzyme is heat
25 labile so the temperature at which cDNA synthesis was
effected had to be within a limited temperature range.
The next step involved destroying the activity of the
RT by heat-inactivation and then adding the DNA
polymerase (PCR step) to initiate the amplification
30 step.

By using a recombinant *Thermus thermophilus* (rTth)
enzyme that possesses both RT activity and DNA

polymerase activity, in the presence of manganese one can reduce and simplify the RT-PCR method to a one-enzyme procedure. Also, using a heat-stable enzyme such as, rTth, one can increase the annealing temperature during the RT stage to reduce the non-specific cDNA generated through non-specific priming, ensuring only the target RNA sequence is transcribed into DNA. This then greatly increases the ability to amplify the target DNA in the next step, the PCR reaction.

Methods of isolating RNA are known, for example Graham et al., (Ref. 1 - various references are referred to in parenthesis to more fully describe the state of the art to which this invention pertains. Full bibliographic information for each citation is found at the end of the specification, immediately preceding the claims. The disclosure of these references are hereby incorporated by reference into the present disclosure). The yields from these methods tend to be very low. When the desired target is a very rare message, these types of isolation procedures do not yield enough total RNA to detect the very rare message. Therefore, it is desirable to improve the yield of the total RNA from various tissue samples so the rare messages are represented. In addition, if the yields of RNA from certain tissues, for example, lungs, are increased, this reduces the need to pool several samples from different animals, thereby reducing the biological diversity. This allows the researcher to define what is happening in a specific animal after treatment.

To investigate the expression levels of rare messages, it is important to extract from the tissue of

interest enough intact total message to enable amplification and quantitation of these rare messages.

SUMMARY OF THE INVENTION

The present invention relates to the determination of RNA production in cells. By modifying existing procedures, the invention permits an accurate quantitation of small quantities of RNA, representative of rare transcripts in tissues or cells, in particular the quantitation of cytokine RNA in mouse lung and spleen tissue.

While other methods of quantitative PT-PCR compare the final PCR product to the level of expression of an endogenous gene or an internal cDNA standard (Refs. 2, 3, 4), the inventors have designed, in one embodiment of the present invention, an RNA standard that binds the same specific primers and probe as the target sequence utilizing a fluorescent tag. This helps equalize the efficiency of the large RT-PCR reaction for the standard RNA and target mRNA in a sample, which are sensitive both to changes in template length and secondary structure. If these reactions are not equivalent, true quantitation cannot be determined.

In accordance with one aspect of the present invention, there is provided a method of determining the quantity of a target RNA in a tissue sample, which comprises isolating the tissue sample from a host; isolating total RNA from the tissue sample; subjecting the isolated RNA to a reverse transcriptase reaction followed by a DNA polymerase amplification reaction (PCR) using primers corresponding to transcribed sequences of the target RNA; binding a labelled sequence corresponding to an internal sequence complementary to one of the strands of the PCR product

of the target RNA; and determining the amount of labelled sequence bound to amplified transcribed target RNA.

In another aspect of the present invention, there is provided a method of quantifying more accurately a target RNA in a tissue sample, which comprises isolating the tissue sample from a host; isolating total RNA from the tissue sample; subjecting the total RNA to a reverse transcriptase reaction followed by a DNA polymerase amplification reaction using primers corresponding to transcribed sequences of the target RNA; binding a labelled sequence corresponding to an internal sequence of the amplified product; and determining the amount of bound labelled sequence by the generation of a detectable label. The detectable label may be fluorescence.

The comparison of the determined amount of bound labelled sequence to an RNA standard may be effected by regression analysis using the plot.

An RNA standard of the number of copies of target RNA may be established. The determined amount of bound labelled sequence in the sample then may be compared to the RNA standard as a measure of the number of copies of target RNA in the tissue sample.

The RNA standard employed in this aspect of the present invention may be established by synthesizing an RNA molecule corresponding to the target RNA; quantifying the synthetic RNA molecule; effecting serial dilution of said synthetic RNA molecule to provide a plurality of samples of known starting copy number; subjecting the synthetic RNA molecule in each sample to a reverse transcriptase reaction followed by a DNA polymerase amplification reaction using primers

- corresponding to transcribed sequences of the synthetic RNA molecule and corresponding in sequence to those employed during DNA polymerase amplification of the transcribed sequence of the target RNA; binding a
- 5 labelled sequence corresponding to an internal transcribed sequence of the synthetic RNA molecule to amplified transcribed synthetic RNA molecule in each sample, the labelled internal sequence being the same as that used to bind to amplified transcribed target
- 10 RNA; determining for each sample the quantity of labelled sequence bound to amplified transcribed target RNA; and plotting the individual determinations of the quantity of labelled sequence in each sample against the log of the known starting copy number for each of
- 15 the samples to provide a plot.

- The detection step may be effected in one of two ways. In one such alternative, the detection step is effected by hybridizing the labelled sequence to the amplified product following the amplification step, and
- 20 including the step of separating bound labelled sequence from unbound labelled sequence prior to the determination step. The label used in this procedure may be radioactive.

- In the other alternative procedure, the detection
- 25 step is effected by effecting the DNA polymerase amplification in the presence of the labelled sequence and the determination of the amount of bound labelled sequence is effected by detecting the generation of a detectable label. The detectable label may be
- 30 fluorescence.

Preferably, the reverse transcriptase and DNA polymerase amplification reactions (RT-PCR) are both carried out using a single enzyme reaction, preferably

using *Thermus Thermophilus* enzyme, which may be found recombinantly.

The detection of the fluorescent signal can be achieved through the use of a real-time detection system, such as described in ref 3.

The primers used in the DNA polymerase reaction may correspond to the 5' and 3' ends of an internal transcribed sequence of the target RNA.

BRIEF DESCRIPTION OF DRAWINGS

Figure 1 shows a construction scheme for the assembly of plasmid pLITMUS-IFN- γ ;

Figure 2 shows the nucleotide sequence for pLITMUS-IFN- γ (SEQ ID NO.9);

Figure 3 shows the nucleotide sequence for the IFN- γ RNA standard (SEQ ID NO. 10);

Figure 4 shows graphically the RNA standard used to determine the quantity of RNA in a sample;

Figure 5 shows optimization of the PCR cycle for single-tube RT-PCR for IL5(a), γ -IFN(b) and IL4(c) a total RNA from mouse lungs; and

Figure 6 shows the quantity of γ -IFN determined in lung samples taken from mice immunized with PBS (#529, 550, 551), live RNA (#549, 509) and formalin-inactivated RSV (#569).

GENERAL DESCRIPTION OF THE INVENTION

RNA isolation may be effected in any convenient manner as long as there is no contamination of genomic DNA which would give a false signal. In one embodiment of the invention, RNA isolation is effected by homogenization of the sample tissue in the presence of TRIzol Reagent (GIBCO/BRL), a mono-phasic solution of phenol and guanidine isothiocyanate. During sample

homogenization or lysis, TRIzol Reagent maintains the integrity of the RNA, while disrupting cells and dissolving cell components. It is important to eliminate any trace organic solvent on the

5 homogenization probe after washing to prevent the DNA migrating from the interphase layer and mixing with RNA in the aqueous phase.

Addition of chloroform to the homogenized sample followed by centrifugation, separates the solution into

10 an organic phase, an interphase layer containing DNA and protein contaminants, and an aqueous phase, where the RNA is exclusively located.

After separation of the aqueous phase, the RNA may be separated therefrom by precipitation through mixing

15 with isopropyl alcohol or other suitable alcohol and centrifugation. The supernatant is removed and the RNA pellet is washed once with ethanol followed by air drying the pellet.

This conventional RNA isolation procedure is

20 improved by washing the homogenization probe between samples in the following manner. The probe first is washed with sterile water then ethanol and then twice with sterile water using wash tubes. The probe is wiped off with a sterile wipe both after the initial

25 and final sterile water wash before proceeding with the homogenizing of the first tissue samples. This washing operation removes residual ethanol and prevents sample carryover which may lead to DNA contamination of the final product.

30 Reverse transcriptase PCR amplification of the RNA is next carried out, preferably, via a single enzyme reaction using rTth DNA polymerase and appropriate primers and probe. The use of such recombinant enzyme

permits higher temperatures for RT incubation, which leads to the generation of more specific DNA.

The gene segments amplified from the transcribed RNA depend on the target RNA under investigation. For example, for murine cytokines, the applicants have identified the following gene segments:

Murine 1L-4: na 249 to 363 (SEQ ID Nos. 1, 2)

Murine 1L-5: na 336 to 402 (SEQ ID Nos. 3, 4)

Murine 1L-10: na 401 to 495 (SEQ ID Nos. 5, 6).

Murine IFN- γ : na 404 to 507 (SEQ ID Nos. 7, 8)

Determination of the quantity of RNA in the specific tissue sample is possible using any convenient label or tag, such as the PE ABI 7700 fluorescence detection system and Taqman chemistry. The use of a fluorescence detection system permits quantitation of RNA in tissue down to a level of 100 molecules.

The determination of the quantity of RNA in the sample is effected, in one embodiment, using an RNA standard, as described above. By first developing a plot of the relationship of the quantity of label to the number of copies of a target RNA in a sample, the unknown quantity of RNA in a given sample can be determined by reading off the value from the plot. The same primers and probe are used both in the development of the standard and in a sample determination to ensure an accurate measure of target RNA in the sample.

The procedure of the present invention enables an improved yield and purity of RNA to be obtained in comparison to published data, such as in the Graham et al reference referred to above. Rather than tissue from two mice being necessary to obtain sufficient RNA

for amplification, individual mice can be analyzed and at least over 200 assays are possible for each RNA sample.

- Whole tissue is analyzed as opposed to RNA
5 extracted from selected cells, as described in Waris et al. above.

SEQUENCES

Mouse IL-4 sequence (sense strand (SEQ ID No: 1) with anti-sense (SEQ ID No: 2) below):

- 10 CGTCCTCACA GCAACGAAGA ACACCACAGA GAGTGAGCTC GTCTGTAGGG CTTCCAAGGT 60
GCTTCGCATA TTTTATTAA AACATGGGAA AACTCCATGC TTGAAGAAGA ACTCT 115
- SEQ ID No. 1
- GCAGGAGTGT CGTTGCTTCT TGTGGTGTCT CTCACCTCGAG CAGACATCCC GAAGGTTCCA 60
CGAAGCGTAT AAAATAAATT TTGTACCCTT TTGAGGTACG AACTTCTTCT TGAGA 115
15 - SEQ ID NO. 2

Mouse IL-5 sequence (sense (SEQ ID No: 3) with anti-sense (SEQ ID No: 4) below):

- ACCGCCAAAA AGAGAAGTGT GCGGAGGAGA GACGGAGGAC GAGGCAGTTC CTGGATTACC 60
TGCAAGA - SEQ ID No: 3 67
- 20 TGGCGGTTTT TCTCTTCACA CCGCTCCTCT CTGCTCCTG CTCGGTCAAG GACCTAATGG 60
ACGTTCT - SEQ ID No: 4 67

Mouse IL-10 sequence (sense strand (SEQ ID No: 5) with anti-sense (SEQ ID No: 6) below).

- TGAATTCCTT GGGTGAAGA CTGAAGACCC TCAGGATGCG GCTGAGGCGC TGTCATCGAT 60
25 TTCTCCCTTG TGAAATAAG AGCAAGGCAG TGGAG - SEQ ID No. : 5 95
- ACTTAAGGGA CCCACTTTC GACTTCTGGG AGTCCTACGC CGACTCCGCG ACAGTAGCTA 60
AAGAGGGGAC ACTTTTATTC TCGTTCCGTC ACCTC - SEQ ID No. : 6 95

Mouse interferon-gamma mRNA sequence (sense strand (SEQ ID No: 7) and anti-sense (SEQ ID No: 8) below):

CATTTCATGAG TATTGCCAAG TTTGAGGTCA ACAACCCACA GGTCCAGCGC CAAGCATTCA 60
ATGAGTCAT CCGAGTGGTC CACCAGCTGT TGCCGGAATC CAGC - SEQ ID NO. : 7 104

GTAAGTACTC ATAACGGTT CAAACTCCAG TTGTGGGTG TCCAGSTCGC GGTCGTAAG 59
TTACTCGAGT AGGCTCACCA GGTGGTCGAC AACGGCCTTA GGTCG - SEQ ID NO. : 8 104

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EXAMPLES

The above disclosure generally describes the present invention. A more complete understanding can be obtained by reference to the following specific Examples. These Examples are described solely for the purposes of illustration and are not intended to limit the scope of the invention. Changes in form and substitution of equivalents are contemplated as circumstances may suggest or render expedient. Although specific terms have been employed herein, such terms are intended in a descriptive sense and not for the purposes of limitations.

Example 1:

This Example describes a general procedure of RNA isolation.

100 mM 2-mercaptoethanol in TRIzol® reagent was prepared. Frozen tissue was homogenized in 1.0 ml 2-ME/TRIzol® per 100 mg tissue with Polytron generator at setting 6 for 50 seconds and 40 seconds, with a 10 second rest in between. Washes of generator probe were performed before beginning the procedure and between each sample. These consisted of homogenization of 30 mL volumes of sterile solutions for 30 seconds at setting 6 with water, 70% ethanol, and two further washes with water. Wiping of the generator probe after the initial and final water wash was performed with a sterile wipe. Additionally, a brief pulse of probe while enclosed

within a wipe was performed at the end of the washes to remove any trapped liquid. After a ten minute incubation at room temperature, the homogenized sample was transferred to a 1.5 mL tube containing 200 μ L 5 chloroform and shaken for 10 seconds. Following a 3 minute incubation at room temperature, the phases were separated by centrifugation at 12,000 x g for 15 minutes at 4°C. 400 μ L of the aqueous (upper) phase was transferred to a 1.5 mL tube containing 330 μ L 10 isopropyl alcohol and vortexed for 10 seconds. After 10 minutes incubation at room temperature, the RNA was pelleted by centrifugation as before. After removal of the supernatant, the pellet was washed with 1.0 mL 75% ethanol. The tube was vortexed for 10 seconds and 15 centrifuged at 7,500 x g for 5 minutes at 4°C. After removal of the supernatant, the RNA pellet was dried for 5 minutes at room temperature. The RNA was dissolved in DEPC-treated water and quantitated by absorbance at 260 nm in a uv spectrophotometer.

20 Example 2:

This Example describes a general procedure single-tube RT-PCR.

cDNA was synthesized from 100 ng of RNA utilizing Mn^{2+} -dependent reverse transcriptase activity of 25 recombinant DNA polymerase rTth from *Thermus thermophilus* and sequence-specific primers. PCR amplification followed in the same tube, also utilizing rTth polymerase. Each RT-PCR was performed in a 20 μ L final volume, with final concentrations of 50 mM Bicine 30 115 mM potassium acetate, 8% (w/v) glycerol, 0.3 mM dNTPs, 0.4 mM primers, 4 U of rTth polymerase, 8 U RNAsin RNase inhibitor, and 2.5 mM $Mn(OAc)_2$, at pH 8.2.

Reverse transcription was performed between 55°C and 65°C for 60 minutes, the actual temperature being optimized for each gene target. The first denaturation was performed at 94°C for 1 minute and the
5 amplification cycles were 94°C for 15 seconds, 55°C for 30 seconds, and 70°C for 30 seconds. The cycle number for each target was optimized to ensure the increase in PCR product was in the exponential growth phase. The results of optimization experiments for IL5(a), γ -
10 IPN(b) or IL4(c) using 25, 30, 35 or 40 cycles are shown in Figure 5.

The RT-PCR products were allowed to hybridize in solution to a ³²P-end-labelled oligonucleotide probe, specific for an internal region of the target sequence.
15 Hybridized fragments were separated from unbound probe using 12.5% poly-acrylamide gel electrophoresis. Dried gels were exposed to X-ray film or scanned for radioactivity on a β -scanner.

Example 3:

20 This Example describes a specific quantitation of γ -interferon expression levels using RT-PCR.

cDNA was synthesized from 100 ng of RNA utilizing Mn²⁺-dependent reverse transcriptase activity of recombinant DNA polymerase rTth from *Thermus*
25 *thermophilus* and sequence-specific primers designed by Primer Express® software. PCR amplification followed in the same tube in the presence of a TaqMan® probe specific for an internal sequence of the target gene, also utilizing rTth polymerase. Each RT-PCR was
30 performed in a 25 μ L-final volume, with final concentrations of 50 mM Bicine, 115 mM potassium acetate, 0.01 mM EDTA, 60 nM passive reference 1(ROX

dye), 8% (w/v) glycerol, 0.3 mM dNTPs, 0.1 to 0.4 mM primers, 5 U of rTth polymerase, 10 U RNasin RNase inhibitor, and 1.0 to 5.0 mM $Mn(OAc)_2$ at pH 8.2 according to manufactures instructions in the TaqMan EZ RT-PCR core reagents (Perkin-Elmer). Reverse transcription was performed at 60°C for 30 minutes. The first denaturation was performed at 95°C for 3 minutes and 40 amplification cycles were as follows: 95°C for 15 seconds, 60°C for 30 seconds, and 70°C for 30 seconds. The RT-PCR products were measured by increases in fluorescent signal above a threshold level (threshold cycle, or C_t). C_t values vary inversely with the amount of nucleic acid in the original sample. Regression analysis was performed using the standard curve of C_t values against log copy number (Figure 4; Example 5), to determine the copy number in each sample based on its experimental C_t value.

Copy number values obtained for mice immunized with PBS (#529, 550, 551), live RSV (#549, 509) or formalin inactivated RSV (#569) are shown in Table I below and plotted graphically in Figure 6.

Example 4:

This Example describes the construction of plasmid pLITMUS-IFN- γ as outlined in Figure 1.

The pLITMUS-IFN- γ plasmid was created by digesting plasmid pMPI3mIF (obtained from Virogenetics Corp.) with the restriction enzymes *HindIII* and *EcoRI* isolating the 624 bp fragment and ligating this fragment into the LITMUS-28 plasmid (New England Biolabs, Mississauga, Ontario, Canada) that had also been cut with *EcoRI* and *HindIII*. The sequence of plasmid pLITMUS-IFN- γ is shown in Figure 2. Plasmid

pMPI3mIF contained a 498 bp cDNA fragment of the IFN- γ mRNA. Although cDNA fragment represents less than half of the IFN- γ mRNA, it includes all of the IFN- γ coding region.

5 Example 5:

This Example describes the preparation of *SpeI* linearized pLITMUS-IFN- γ required for the generation of the IFN- γ RNA standard in an *in vitro* transcription reaction and the preparation of the IFN- γ RNA standard.

10 20 μ g of pLITMUS-IFN- γ DNA was cut with *SpeI* in a reaction containing 20 mM Tris-HCl (pH 7.4), 5 mM MgCl₂, 50 mM KCl and 80 units of *SpeI* (Life Technologies, Gaithersburg, MD, USA). The nucleotide sequence of the linearized plasmid is shown in Figures 2 (SEQ ID No.9).

15 IFN- γ RNA was generated from 2 μ g of the linearized plasmid in a 50 μ L *in vitro* transcription reaction using the following materials:

40 mM Tris-HCl (pH 8.0)

8 mM MgCl₂

20 2 mM spermidine - (HCl),

20 mM DTT (dithiothreitol)

1 mM ATP (adenosine triphosphate)

1 mM GTP (guanosine triphosphate)

1 mM CTP (cytidine triphosphate)

25 1 mM UTP (uridine triphosphate)

100 units of RNasin® RNase inhibitor (Promega, Madison, WI, USA)

250 units of T7 RNA polymerase (Life Technologies, Gaithersburg, MD, USA).

30 The reaction was incubated at 37°C for 60 minutes. To digest the DNA template, 5 units of *DNaseI* was added

to the reaction which was then incubated a further 15 minutes at 37°C. The IFN- γ RNA standard was then purified away from the salt, enzymes, unincorporated NTP's and digested DNA fragments by passing the reaction mix through a CHROMA SPINTM-30 DEPC-H₂O columns (Clontech, Palo Alto, CA, USA) according to the manufacturer's instructions.

The IFN- γ RNA standard was then quantified by measuring its OD at a wavelength of 260 nm (1 OD at 260 nm = 40 μ g of RNA). Since the RNA has a length of 738 bases and each base has a molecular weight (MW) of 340 daltons, the MW of this RNA standard can be calculated (738 X 340 = 250,920 daltons). If the mass of the RNA is divided by its MW, the number of moles (e.g. 1 μ g of the RNA standard contains 1×10^{-6} g/250,920 daltons = 3.9853×10^{-12} moles or 3.9853 pmols) can be determined. Since 1 mole of any compound contains 6.022×10^{23} molecules, the number of copies of RNA that are in 1 μ g of the IFN- γ RNA standard can be determined (1 μ g contains 3.9853×10^{-12} moles X 6.02×10^{23} = 2.3999×10^{12} copies of the RNA standard per 1 μ g). The standard can then be diluted to give any copy number desired in each reaction. The nucleotide sequence of the IFN- γ RNA standard transcript is shown in Figure 3 (SEQ ID No. 10).

Log dilutions of the RNA standard were used to prepare a curve of threshold values (Ct) plotted against log input copy number and is shown in Figure 4.

SUMMARY OF THE DISCLOSURE

In summary of this disclosure, the present invention provides an accurate method of determining the quantity of specific RNA in cells, to permit

analysis of rare transcripts, such as cytokines, based on a modified RNA isolation procedure, RT-PCR in a single enzyme reaction, detection and comparison to a RNA standard. Modifications are possible within the

5 scope of this invention.

TABLE I

Sample	Immunization	Copy #	+/-SD
529	PBS	305.82	66.67
549	Live RSV	15704.12	1575.72
569	FI RSV	7328.56	799.68
509	Live RSV	15399.17	818.21
550	PBS	1873.54	328.59
551	PBS	2732.02	346.20

FI - Formalin inactivated RSV

PBS - Phosphate buffered saline

REFERENCES

1. Graham BS, Henderson GS, Tang Y-W, Lu X, Neuzil KM, Colley DG. Priming immunization determines T helper cytokine mRNA expression patterns in lungs of mice challenged with respiratory syncytial virus. *J Immunol* 1994; 151:2032-2040.
2. Heid CA, Stevens J, Livak KJ, Williams PM. Real time quantitative PCR. *Genome Res* 1996; 6:986-994.
3. Gibson UEM, Heid CA, Williams PM. A novel method for real time quantitative RT PCR. *Genome Res* 1996; 6:995-1001.
4. Wang, AM, Doyle, MV, Mark, DF. Quantitation of mRNA by the polymerase chain reaction. *Proc. Natl. Acad. Sci.* 1989; 86:9717-9721.
5. Waris, ME, Tsou, C, Erdman, DD, Zaki, SF, Anderson, L. Respiratory Syncytial Virus Infection in Balb/c Mice Previously Immunized with Formalin-Inactivated Virus Induces Enhanced Pulmonary Inflammatory Response with a Predominant Th2-Like Cytokine Pattern. *J Virol* 1996; 70 (5):2852-2600.
6. Siebert, PD, Chenchik, A. Modified acid guanidinium thiocyanate-phenol-chloroform RNA extraction method which greatly reduces DNA contamination. *Nucleic Acids Res* 1993; 21(8):2019-2020.

CLAIMS

What we claim is:

1. A method of determining the quantity of a target RNA in a tissue sample, which comprises:
 - isolating the tissue sample from a host,
 - isolating total RNA from the tissue sample,
 - subjecting the isolated RNA to a reverse transcriptase reaction followed by a DNA polymerase amplification reaction using primers corresponding to transcribed sequences of the target RNA,
 - binding a labelled sequence corresponding to an internal transcribed sequence of the target RNA to amplified transcribed target RNA,
 - determining the amount of labelled sequence bound to amplified transcribed target RNA,
 - establishing an RNA standard of the number of copies of the target RNA, and
 - comparing the determined amount of bound labelled sequence in the sample to the RNA standard as a measure of the number of copies of target RNA in the tissue sample.
2. The method of claim 1 wherein said RNA standard is established by:
 - synthesizing an RNA molecule corresponding to the target RNA,
 - quantifying the synthetic RNA molecule,
 - effecting serial dilution of said synthetic RNA molecule to provide a plurality of samples of known starting copy number,
 - subjecting the synthetic RNA molecule in each sample to a reverse transcriptase reaction followed by a DNA polymerase amplification reaction using primers corresponding to transcribed sequences of the synthetic

RNA molecule and corresponding in sequence to those employed during DNA polymerase amplification of the transcribed sequence of the target RNA,

binding a labelled sequence corresponding to an internal transcribed sequence of the synthetic RNA molecule to amplified transcribed synthetic RNA molecule in each sample, said labelled internal sequence being the same as that uses to bind to amplified transcribed target RNA,

determining for each sample the quantity of labelled sequence bound to amplified transcribed target RNA, and

plotting the individual determination of the quantity of labelled sequence in each sample against the log of the known starting copy number for each of the samples to provide a plot.

3. The method of claim 2 wherein said comparison of the determined quantity of bound labelled sequence to the RNA standard is effected by regression analysis using said plot.

4. The method of claim 3 wherein said binding step is effected by hybridizing said labelled sequence to said amplified transcribed target RNA following said amplification step, and including the step of separating bound labelled sequence from unbound labelled sequence prior to said determination step.

5. The method of claim 4 wherein said label is radioactive.

6. The method of claim 3 wherein said binding step is effected by effecting said DNA polymerase amplification in the presence of said labelled sequence and said determination of the amount of bound labelled sequence

is effected by detecting the generation of a detectable label.

7. The method of claim 6 wherein said detectable label is fluorescence.

8. The method of claim 1 wherein said reverse transcriptase reaction and said DNA polymerase amplification reaction are both carried out using a single enzyme reaction.

9. The method of claim 8 wherein said single enzyme reaction is effected using *Thermus thermophilus* enzyme.

10. The method of claim 1 wherein said target RNA is a cytokine.

11. The method of claim 1 wherein said primers correspond to an internal transcribed sequence of the target RNA.

12. A method determining the quantity of a target RNA in a tissue sample, which comprises:

isolating the tissue sample from a host;

isolating total RNA from the tissue sample;

5 subjecting the isolated RNA to a reverse transcriptase reaction followed by a DNA polymerase amplification reaction (PCR) using primers corresponding to transcribed sequences of the target RNA;

10 binding a labelled sequence corresponding to an internal sequence complementary to one of the strands of the PCR product of the target RNA; and determining the amount of labelled sequence bound to amplified transcribed target RNA.

13. A method of quantifying more accurately a target RNA in a tissue sample, which comprises:

isolating the tissue sample from a host;

isolating total RNA from the tissue sample;

subjecting the total RNA to a reverse transcriptase reaction followed by a DNA polymerase amplification reaction using primers corresponding to transcribed sequences of the target RNA;

binding a labelled sequence corresponding to an internal sequence of the amplified product; and determining the amount of bound labelled sequence by the generation of a detectable label.

14. The method of claim 13 wherein the detectable label is a fluorescent tag.

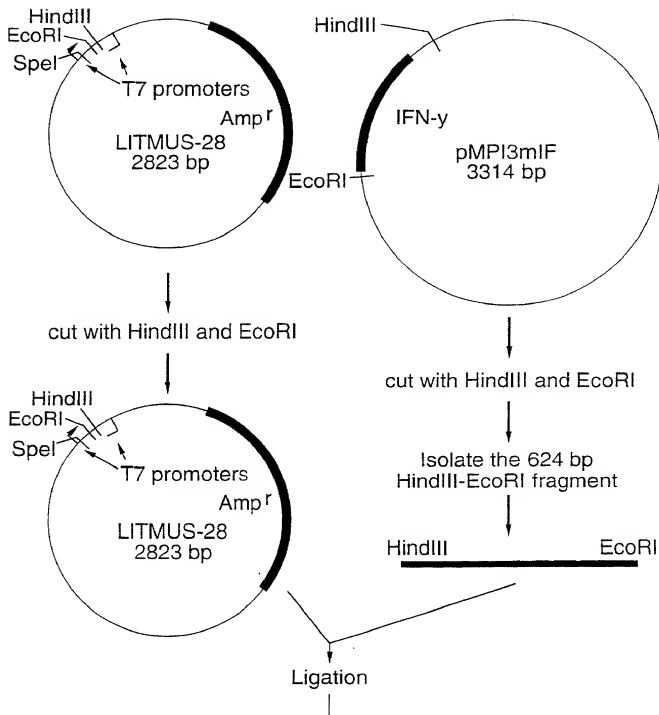
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Construction of pLITMUS-IFN- γ 

FIG.1A

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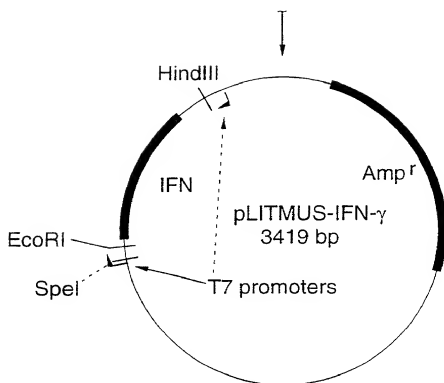


FIG.1B

FIG.2A

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Sequence of pLITMUS-IFN- γ

GTTAACTACG TCAGGTGGCA CTTTTCGGGG AAATGTGGCG GGAACCCOCTA TTGTGTTTATT
 TTTCFAAATA CATTCAAATA TGTATCCGCT CATGAGACAA TAACCCCTGAT AAATGCTTCA
 ATAATATTGA AAAAGGAAGA GTATGAGTAT TCAACATTTC CGTGTGCGCC TTATTCCCTTT
 TTTTGGCGCA TTTTGGCTTC CTGTTTTTCG TCACCCAGAA ACGCTGGTGA AAGTAAAAGA
 TGCTGAAGAT CAGTTGGGTG CACGAGTGGG TTACATCGAA CTGGATCTCA ACAGCGGTAA
 GATCCTTGAG AGTTTTCGGC CCGAAGAACG TTCTCCAATG ATGAGCACTT TTAAAGTTCT
 GCTATGTGGC GCGGTATTAT CCCGTGTGTA CGCGGGGCAA GAGCAACTCG GTCCCGGCAT
 ACACTATTCT CAGAATGACT TGGTTGAGTA CTCACCAGTC ACAGAAAAGC ATCTTACGGA
 TGGCATGACA GTAAGAGAAT TATGCAGTGC TGOCATAACC ATGAGTGATA ACACTGCGGC
 CAACTTACTT CTGACAACGA TCGGAGGACC GAAGGAGCTA ACCGCTTTTT TGCACAACAT
 GGGGGATCAT GTAACTCGCC TTGATCGTTG GGAACCGGAG CTGAATGAAG CCATACCAAA
 CGACGAGGCT GACACACGCA TGCCCTGAGC AATGGCAACA ACGTGTGGCA AACTATTAAAC
 TGGCGAAGTA CTTACTCTAG CTTCCCGGCA ACAATTAATA GACTGGATGG AGGCGGATAA
 AGTTGCAGGA CCACTTCTGC GCTCGGCCCT TCCGGCTGGC TGGTTTATTG CTGATAAATC
 TGGAGCCGGT GAGCGTGGGT CTCGCGGTAT CATTCGACGA CTGGGGCCAG ATGGTAAGCC
 CTCGCGTATC GTAGTTATCT ACACGACGGG GAGTCAGGCA ACTATGGATG AACGAATAG
 ACAGATCGCT GAGATAGGTG CCTCACTGAT TAAGCATTGG TAACGTTCAG ACCACTTTTA
 CTCATATATA CTTTATAGTTG ATTTACCCCG GTTGATAATC AGAAAAGGCC CAAAAACAGG
 AAGATGTGAT AAGCAATAT TTAANTTGTA AACGTTAATA TTTTGTAAAA ATTCGCGTTA
 AATTTTTGTT AAATCAGCTC ATTTTTTAAC CAATAGCCCG AAATCGGCAA AATCCCTTAT
 AAATCAAAAG AATAGCCCGA GATAGGGTTG AGTGTGTTC CAGTTTGGAA CAAGAGTCCA
 CTATTAAGA ACGTGGACTC CAACGTCAA GGGCGAAAA CCGCTATCA TGGCGATGCG
 CCACTACGTC AACCATCACC CAAATCAAGT TTTTGGGGT CGAGTGTCCG TAAAGCACTA
 AATCGGAACC CTAAAGCGAG CCCCAGATTG AGAGCTTGAC GGGGAAAGCG AACGTGGCGA
 GAAAGGAAGG GAAGAAAGCG AAAGGAGCGG GCGCTAGGGC GCTGGCAAGT GTAGCGGTCA
 CGCTGCGGCT AACCACCACA CCGCGCGGCG TTAATCGGCC GCTACAGGCG GCGTAAAGAG
 ATCTAGGTGA AGATCCCTTT TGATAATCTC ATGACCAAAA TCCCTTAACG TGAGTTTTCG
 TTCCACTGAG CGTCAGACCC CGTAGAAAA ATCAAAGGAT CTCTTGAGA TCTTTTTTTT
 CTGCGGTGTA TCTGCTGCTT GCAAAACAAA AAACCAACCG TACCAGCGGT GGTTTGTTTG
 CCGGATCAG AGCTACCAAC TCTTTTTCCG AAGGTAAC TGCTTACGAG AGCGCAGATA
 CCAAATACTG TTTCTCTAGT GTAGCCGTAG TTAGGCCACC ACTTCAAGAA CTCTGTAGCA
 CCGCTACAT ACCTCGCTCT GCTAATCCCTG TTACAGTGG CTGCTGCCAG TGGCGATAAG
 TCGTGTCTTA CCGGTTTGA CTCAAGACGA TAGTTACCGG ATAAGGCGCA GCGGTGGGGC
 TGAACGGGGG GTTCGTGCAC ACAGCCAGC TTGGAGCGAA CGACCTACAC CGAACTGAGA
 TACCTACAG GTGAGCTATG AGAAAGCGCC ACGCTTCCCG AAGGGAGAAA GCGGGACAGG
 TATCCGTTAA CCGGCAGGGT CGGAACAGGA GAGCGCACGA GGGAGCTTCC AGGGGGAAAC

FIG.2B

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GCGTGGTATC TTATAGTCC TGTCGGGTTT CGCCACCTCT GACTTGAGCG TCGATTTTTTG
 TGATGCTCGT CAGGGGGGCG GAGCCTATGG AAAAAAGGCC GCAACGGGGC CTTTTTACGG
 TTCTCGGGCT TTTGCTGGCC TTTTGCTCAC ATGTAATGTG AGTTAGCTCA CTCATTAGGC
 ACCCCAGGCT TTACACTTTA TGCTTCCGGC TCGTATGTTG TGTGGAATTG TGAGCGGATA
 ACAATTTCAC ACAGGAAACA GCTATGACCA TGATTACGCG AAGCTACGTA ATACGACTCA
 CTAGTGGGCA GATCTTOGAA TGCACTCGCG GCACCGTAGC TCTOGAGGAA TTCCCGGGGA
 TCCCTGCAGT TATTGGGACA ATCTCTTCCC CACCCGGAAT CAGCAGCGAC TCCTTTTCCG
 CTTCCTGAGG CTGGATTCGG GCAACAGCTG GTGGAOACT CGGATGAGCT CATTGAATGC
 TTGGCGCTGG ACCTGTGGGT TGTTGACCTC AAACCTGGCA ATACTCATGA ATGCATCCTT
 TTTGCGCTTG CTGTTGCTGA AGAAGGTAGT AATCAGGTGT GATTCAATGA CGCTTATGTT
 GTTGCTGATG GCGTGATTGT CTTTCAAGAC TTCAAAGAGT CTGAGGTAGA AAGAGATAAT
 CTGGCTCTGC AGGATTITCA TGTCACCATC CTTTGTCCAG TTCCTCCAGA TATCCAAGAA
 GAGACTCTTT TCTTCCACAT CTATGCCACT TGAGTTAAAA TAGTTATCA GACTTTCTAG
 GCTTTCAATG ACTGTGCCGT GGCAGTAACA GCCAGAAACA CCCATGAGGA AGAGCTGCAA
 AGCCAAGATG CAGTGTGTAG CGTTCATGAT TAGATTAAAC TAAATAATTG TACTTTGTAA
 TATAATGATA TATATTTTCA CTTTATCTCA TTTGAGAATA AAAATGTTTT TGTTTAACCA
 CTGCATGATG TAAGCTTCCC ATGGTGACGT CACCGGTTCT AGATACCTAG GTGAGCTCTG
 GTACCTCTTA GTCAAGGCGT TAAGTGAGTC GTATTACGGA CTGGCCGCTG TTTTACAACG
 TCGTGACTGG GAAAACCTG GCGTTACCCA ACTTAATCGC CTTGCAGCAC ATCCCCCTTT
 CGCCAGCTGG CGTAATAGCG AAGAGGCCCG CACCGATCGC CCTTCCCAAC AGTTGCGCAG
 CCTGAATGGC GAATGGCGCT TCGCTTGGTA ATAAAGCCCG CTTGCGCGGG CTTTTTTTTT

Utilized T7 promoter 3199 - 3216

Transcription start site 3198

IFN- γ specific sequence 2530 - 3027

Transcript sequence 2464 - 3198

SpeI site used for linearization 2461

FIG.3A

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Sequence of the IFN- γ RNA Standard

1 GCCUUGACUA GAGGGUACCA GAGCUCACCU AGGUAUCUAG AACCGGUGAC 50
51 GUCACCAUGG GAAGCUACA UCAUGCAGUG GUAAACAAA AACAUUUUA 100
101 UUCUCAAUG AGAUAAAGUG AAAAUUAUA UCAUUAUAU ACAAAGUACA 150
151 AUUAUUUAGG UUAUUCUAAU CAUGAACGCU ACACACUGCA UCUUGGCUU 200
201 GCAGCUCUUC CUCAUGGCUG UUUCUGGCUG UUAUGCCAC GGCACAGUCA 250
251 UUGAAAGCCU AGAAAGUCUG AAUAACUAU UUAACUCAAG UGGCAUAGAU 300
301 GUGGAAGAAA AGAGUCUCU CUUGGAUAUC UGGAGGAACU GGCAAAAGGA 350
351 UGGUGACAUG AAAAUCCUGC AGAGCCAGAU UAUCUCUUUC UACCCAGAC 400
401 UCUUGAAGU CUUGAAAGAC AAUCAGGCCA UCAGCAACAA CAUAAGCGUC 450
451 AUUGAAUCAC ACCUGAUUAC UACCUUCUUC AGCAACAGCA AGGCGAAAAA 500
501 GGAUGCAUUC AUGAGUAUUG CCAAGUUUGA GGUCAACAAC CCACAGGUCC 550
551 AGCGCCAAGC AUUCAUAGAG CUCAUCCGAG UGGUCCACCA GCTGUUGCCG 600
601 GAAUCCAGCC UCAGGAAGCG GAAAAGGAGU CGCUGCUGAU UCGGGGUGGG 650
651 GAAGAGAUUG UCCCAUAUAC UGCAGGAUC CC CGGGAUU CCTUGAGAGC 700
701 UACGGUGCGC GCGAUGCAU CGAAGAUUC CCCACUAG 738

IFN- γ specific sequence 172 - 669

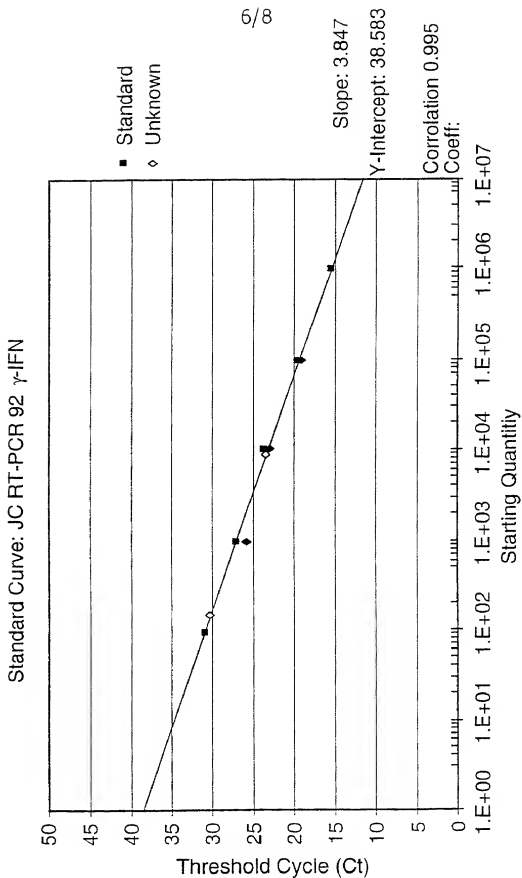


FIG.4

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RT-PCR Cycle Optimization for Lialuid Hybridization

PCR Cycle Optimization for IL5 Amplification

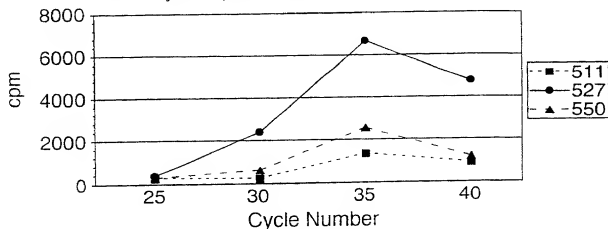


FIG.5A

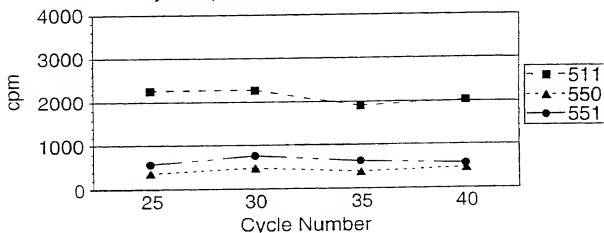
PCR Cycle Optimization for γ -IFN Amplification

FIG.5B

PCR Cycle Optimization for IL4 Amplification

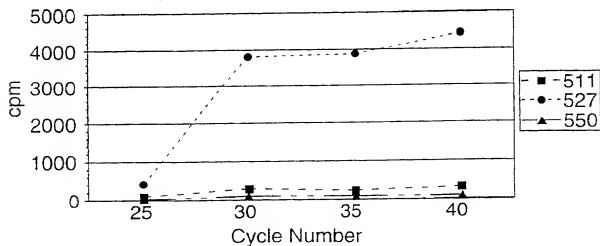
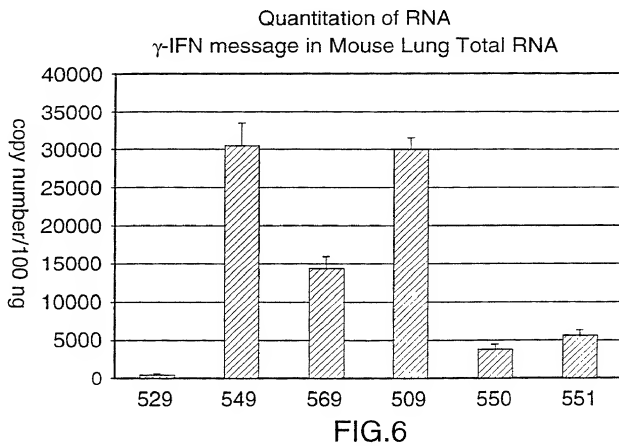


FIG.5C

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Docket No.
1038-1130 MIS:jb

Declaration and Power of Attorney For Patent Application

English Language Declaration

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name,

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled

QUANTITATION OF RNA

the specification of which
(check one)

☐ is attached hereto.

☒ was filed on October 4, 1999 as United States Application No. or PCT International

Application Number PCT/CA99/00917

and was amended on _____

(if applicable)

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose to the United States Patent and Trademark Office all information known to me to be material to patentability as defined in Title 37, Code of Federal Regulations, Section 1.56.

I hereby claim foreign priority benefits under Title 35, United States Code, Section 119(a)-(d) or Section 365(b) of any foreign application(s) for patent or inventor's certificate, or Section 365(a) of any PCT International application which designated at least one country other than the United States, listed below and have also identified below, by checking the box, any foreign application for patent or inventor's certificate or PCT International application having a filing date before that of the application on which priority is claimed.

Prior Foreign Application(s)

Priority Not Claimed

(Number)

(Country)

(Day/Month/Year Filed)

☐

(Number)

(Country)

(Day/Month/Year Filed)

☐

(Number)

(Country)

(Day/Month/Year Filed)

☐

I hereby claim the benefit under 35 U.S.C. Section 119(e) of any United States provisional application(s) listed below:

60/103,153

October 5, 1998

(Application Serial No.)

(Filing Date)

(Application Serial No.)

(Filing Date)

(Application Serial No.)

(Filing Date)

I hereby claim the benefit under 35 U. S. C. Section 120 of any United States application(s), or Section 365(c) of any PCT International application designating the United States, listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States or PCT International application in the manner provided by the first paragraph of 35 U.S.C. Section 112, I acknowledge the duty to disclose to the United States Patent and Trademark Office all information known to me to be material to patentability as defined in Title 37, C. F. R., Section 1.56 which became available between the filing date of the prior application and the national or PCT International filing date of this application:

PCT/CA99/00917

October 4, 1999

(Application Serial No.)

(Filing Date)

(Status)
(patented, pending, abandoned)

(Application Serial No.)

(Filing Date)

(Status)
(patented, pending, abandoned)

(Application Serial No.)

(Filing Date)

(Status)
(patented, pending, abandoned)

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

POWER OF ATTORNEY: As a named inventor, I hereby appoint the following attorney(s) and/or agent(s) to prosecute this application and transact all business in the Patent and Trademark Office connected therewith. (list name and registration number)

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
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Sixth inventor's signature	Date
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